Pathologic gene expression in Gaucher disease: up-regulation of cysteine proteinases including osteoclastic cathepsin K

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Deficiency of lysosomal acid β-glucosidase induces glycolipid storage in the macrophages of Gaucher disease but the pathways of multisystem tissue injury and destruction are unknown. To investigate the cognate molecular pathology of this inflammatory disorder, genes that were differentially expressed in spleen samples from a patient with Gaucher disease (Gaucher spleen) were isolated. Of 64 complementary DNA (cDNA) fragments sequenced from an enriched Gaucher cDNA library, 5 encode lysosomal proteins (cathepsins B, K, and S, α-fucosidase, and acid lipase), 10 encode other known proteins, and 2 represent novel sequences from human macrophage cell lines. Transcript abundance of the cathepsins, novel genes, pulmonary and activation-regulated chemokine (PARC), and NMB, a putative tumor suppressor gene, was greatly increased. Immunoblotting showed increased mature forms of all 3 cathepsins found in samples of Gaucher spleens. Immunofluorescence microscopy showed strong cathepsin B and K reactions in sinusoidal endothelium and Gaucher cells. The respective means, plus or minus SD, of cathepsin B, K, and S activities were 183 ± 35, 97 ± 39, and 91 ± 45 nmol/min/mg protein in 4 Gaucher spleens, and 26 ± 4, 10.5 ± 2, and 4.0 ± 2.1 nmol/min/mg protein in 3 control spleens. Plasma cathepsin B, K, and S activities were also elevated in Gaucher disease plasma (P < .001), but compared with control plasma samples, neither cathepsin B nor K activities were significantly elevated in 8 patients with non-glycosphingolipid lysosomal storage diseases or in 9 patients with other glycosphingolipidoses, which suggests disease specificity. All 3 cathepsin activities were increased 2-fold to 3-fold in Gaucher sera compared with control sera. In all 6 patients treated by enzyme replacement for 16–22 months, serum cathepsin activities decreased significantly (P < .01). Longitudinal studies confirmed the progressive reduction of proteinase activities during imiglucerase therapy but in 3 Gaucher patients with mild disease not so treated, serum cathepsin activities remained constant or increased during follow-up. Enhanced expression of cysteine proteinases may promote tissue destruction. Moreover, the first identification of aberrant cathepsin K expression in hema-topoietic tissue other than osteoclasts implicates this protease in the breakdown of the matrix that characterizes lytic bone lesions in Gaucher disease. (Blood. 2000; 96:1969-1978)

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Introduction

Lysosomal storage diseases result from inborn defects of acid hydrolases or membrane transporters that lead to the pathologic accumulation of complex cellular macromolecules. Gaucher disease, a multisystem disorder affecting macrophages, is identified in the Online Mendelian Inheritance in Man (OMIM) website as OMIM 23080, 23091, and 23100, has become the prototype of the glycosphingolipidoses, an important group of lysosomal disorders. The condition is caused by inherited deficiency of the acid β-glucosidase, glucocerebrosidase (Enzyme Commission [EC] 3.2.1.45), which is widely distributed in the lysosomes. The principal storage material in Gaucher disease is N-acyl-sphingosyl-1-O-β-D-glucoside, but other minor components, including glucosyl sphingosine, also accumulate. These glycolipids of membrane gangliosides and globosides. In the nonneuronopathic (type I) form of Gaucher disease, partial deficiency of glucocerebrosidase is associated with the accumulation of glycolipids in the mononuclear phagocyte system, especially the liver, bone marrow, and spleen. Here the stored material originates from the turnover of exogenous lipids derived from the breakdown of blood cells by macrophages; indeed the pathognomonic Gaucher cell that characterizes this disorder is of the macrophage lineage. Severe deficiency of glucocerebrosidase caused by disabling mutations is additionally associated with neurologic manifestations: failure to degrade endogenous glycosphingolipids present in brain tissue leads to the neuronopathic (type II and III) disease variants.

Although the Gaucher cell is a striking histologic feature of Gaucher disease, the relationship between the lysosomal storage of glycosphingolipid and the protease manifestations of this disorder are unexplained. The disease is accompanied by weight loss, fatigue, increased metabolic rate, pungueculae, and a sustained acute inflammatory reaction with polyclonal or monoclonal B-cell proliferative responses. These manifestations accompany massive enlargement of the spleen and liver; bone infarction crises; and osteolytic lesions as well as tissue injury in the liver, lung, bone marrow, and brain stem. Although the liver and spleen are the main sites for the accumulation of...
glycolipid in the body and may increase to a mass more than 4-fold and 80-fold greater than normal, respectively, the pathologic lipid accounts for less than 2% of the additional tissue weight. Thus the connection between the macrophage abnormality and the complex inflammatory phenotype that characterizes this disease remains obscure. It seems likely that the accumulated glycolipid activates macrophages to induce an inflammatory response. Hence, factors released by Gaucher cells may provide a mechanistic link between the lysosomal storage and the clinical manifestations of established Gaucher disease.

To understand better the pathogenesis of Gaucher disease, we examined the molecular characteristics of the disorder by studying the gene expression profile of the Gaucher cell within its tissue context. We used a newly described subtractive procedure based on the polymerase chain reaction (PCR) to identify genes whose transcription products are increased in Gaucher disease tissue. The method overcomes the problem of differences in the distribution of gene transcripts in the 2 populations by incorporating a hybridization step that normalizes complementary DNA (cDNA) abundance within each population. We report the identification of multiple genes, including a chemokine and others associated with the inflammatory response whose expression is enhanced in Gaucher disease; 5 lysosome protein-specific cDNAs were also identified. Here we focus on overexpression of the lysosomal cysteine proteases, cathepsins B, K, and S, which are now known to participate in tissue modeling, antigen presentation, and, in the case of cathepsin K, bone matrix destruction.

Patients, materials, and methods

Patients

Samples of serum were collected from 12 patients with type 1 Gaucher disease who were attending a specialist center; the serum was taken with patient consent during the course of routine clinical monitoring. Gaucher disease was diagnosed on the basis of histologic and enzymatic criteria. Serum samples were also collected from 26 healthy control subjects, none of whom had a history of arthritis or Gaucher disease. The mean age of the patients with Gaucher disease was 48 years (range, 29-71 years). Of these 12 patients, 5 were men and 7 were women; 2 of the women and 3 of the men had undergone prior splenectomy. The age range of the control subjects, 12 men and 14 women, was 19-60 years.

Venous blood was collected from each subject and allowed to clot. It was centrifuged at 2000g for 10 minutes to separate the serum, and aliquots were transferred to polypropylene Eppendorf tubes and frozen at −70°C before assay. Plasma samples obtained from heparinized blood were also collected at the time of diagnosis from 11 patients with enzymatically confirmed Gaucher disease, 8 patients with nonglycolipid storage disorders, 9 patients with glycosphingolipidoses other than Gaucher disease, and 21 healthy control subjects. The experimental protocol was reviewed and approved by the Cambridge Research Ethics Committee of the East Anglian Health Authority at Addenbrooke's Hospital, Cambridge, England. Radiological lesions, indicating skeletal complications of Gaucher disease, were evident in 5 patients.

Tissue samples

Fresh splenic tissue was diced into 1-cm cubes and frozen in liquid nitrogen within a few minutes of removal; care was taken to use sections of tissue that were neither infarcted nor necrotic, as confirmed by histologic examination. Tissue integrity and polypeptide content were monitored in these samples using protein polyacrylamide gel electrophoresis (PAGE). Therapeutic splenectomy was carried out in these patients if they had intractable cytopenia, hypersplenism, or marked pressure symptoms. In 2 of 4 patients, enzyme replacement therapy in the form of human placental mannosyl-terminated glucerase (Ceredase-alglucerase) had been administered at a dose of 40 U/kg/mo for 6 months without hematologic improvement. Macroscopic inspection of splenectomy specimens showed a large single area of recent infarction and necrosis. The material used for a subsequent study was sampled from tissue remote from these areas of disease. Control spleen samples were obtained from 3 individuals: a 32-year-old man with T-cell lymphoma and associated hypersplenism; a 52-year-old man suffering from idiopathic thrombocytopenic purpura (ITP) refractory to corticosteroids; and a 14-year-old male suffering from a recessive variant of hereditary spherocytosis.

Reagents and laboratory materials

We obtained the following materials: nitrocellulose filters (Schleicher and Schuell); α-32P-dCTP (cytidine 5′-triphosphate) (Amersham International, Little Chalfont, England) with a specific activity of 11.1 × 106 Ci/mmol; RNA markers (Promega Company, Charbonnieres, France); prelabeled molecular weight protein markers and precast sodium dodecyl sulfate (SDS)-PAGE (Bio-Rad Laboratories, Hercules, CA); fluorogenic substrates N-carbobenzyloxy (CBZ)-phenylalanine-arginine-7-amido-4-methylcoumarin (AMC) (CBZ-F-R-AMC) and CBZ-R-R-AMC (Sigma Chemical, Poole, England); and antibodies to cathepsin B (Sorotec, Oxford, England). All other biochemicals were of the highest purity commercially available for molecular biology use. The proteins were stained with Coomassie R250 blue. Rabbit antiserum to human cathepsin K was prepared as reported by Sukhova et al.

Isolation of RNA

Total RNA was isolated from approximately 100 mg frozen splenic tissue using the Trizol reagent (Gibco BRL Life Technologies, Ergany, France) according to the manufacturer’s instructions. Poly (A+)-RNA was purified by the use of oligo d(T) spin columns (R & D Systems Europe, Abingdon, England).

Subtractive hybridization

A subtracted cDNA library of spleen cells from a patient with Gaucher disease (Gaucher spleen) was constructed using a minor modification of the PCR Select cDNA subtraction system (Clontech Laboratories, Heidelberg, Germany) using RNA prepared from the ITP control and a single Gaucher disease (Gaucher spleen) was constructed using a minor modification of the PCR Select cDNA subtraction system (Clontech Laboratories, Heidelberg, Germany) using RNA prepared from the ITP control and a single Gaucher spleen cell.

A double-stranded cDNA population was obtained from the Gaucher spleen, and a reference population of cDNAs was obtained from the control spleen by reverse transcription using oligo d(T) primers and amplification of RNA polymerase I. These were digested with RsaI, a restriction endonuclease. Pools of the Gaucher spleen cDNA in separated tubes were ligated to separate adaptor molecules overnight at 16°C. The sequences of these oligonucleotides and of the oligonucleotide primers subsequently employed in PCR-based amplification reactions are set out at the end of this section (see below). We mixed 20 ng ligated Gaucher spleen cDNA and 600 ng nonligated control spleen cDNA and the samples were denatured by heating to 98°C for 90 seconds. These samples were allowed to anneal for 10 hours at 68°C. After this first hybridization, the 2 samples were combined, and a fresh portion of approximately 150 ng of pooled heat-denatured control spleen cDNA was added. These samples were allowed to hybridize for a further 16 hours at 68°C.
using the same conditions as described in the primary amplification. The only exception was that the oligonucleotide primer P1 was replaced with the following internally orientated and nested PCR oligonucleotide primers: adapter 1, 5'-CTAATACGACTCACTATAGGGTCGACGCGCCGC- CCGGACAGTT-3', adapter 2, 5'-CTAATACGACTCACTATAGGGACCGTGGTATCGGGAGG- CTTAGGCTGCGGGCTGAGTT-3'; PCR primer 1, 5'-CTAATACGACTCACTATAGGGACCGTGTT ATCGGGAGG-3'; nested PCR primer 1, 5'-TCAAGGCGCGCGCCCGGCAGTT-3'; and nested PCR primer 2, 5'-AGCGGTTCGCGCCAGAGTT-3'.

Cloning and analysis of subtracted cDNA populations

The products of the secondary amplification reactions were ligated into the pT-Adv vector (Clontech). Approximately 150 ng PCR-amplified cDNA were ligated into 50 ng of the vector and the ligation mixture was incubated at 37°C until small colonies became visible; they were then further incubated at 4°C until blue-white staining ranoside. The plates were incubated at 37°C until plasmid DNA inserts were sequenced by single-pass fluorescent dye

Immunoblotting. For electrophoresis, 10 μg protein from each extract was denatured in solubilizing buffer containing 62.5 mM/L Tris HCl, 2% wt/vol SDS, 100 mM/L dithiothreitol, and 10% wt/vol glycerol containing 0.025% bromophenol blue (pH 6.8). The protein standards (Bio-Rad) were run in parallel. The samples were electrophoresed in 12% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane electro-photorecically at 100 V for 90 minutes in 25 mM/L Tris, 190 mM/L glycine, and 20% wt/vol methanol (pH 8.3). The membrane was then incubated for 2 hours in a blocking solution containing 5% wt/vol dried milk, 5% wt/vol bovine serum albumin (BSA), 0.1% wt/vol Triton X-100, and 0.1% sodium deoxycholate.

This was followed by a sequential incubation with anticathepsin B (dilution, 1:500) followed by biotin-conjugated antitissue (dilution, 1:1000) or antirabbit immunoglobulin G (IgG) (Sigma) and avidin-alkaline phosphatase (dilution, 1:500) (Sigma). Each incubation was carried out for 2 hours at room temperature, and the membrane was washed 3 times with phosphate-buffered saline (PBS) and 0.2% wt/vol Tween 20 between each change of reagent wash. The membrane was incubated with 0.1 mol/L ethanolamine HCl (pH 9.6) for 10 minutes and then with substrate solution comprising 100 μg/mL 5-bromo-chloro-indolyl-phosphate, 100 μg/mL nitroblue tetrazolium, 1 mM/L magnesium dichloride (MgCl2), and 0.1 mol/L ethanolamine (pH 9.6). The reaction was terminated by rinsing the membrane with distilled water. The same procedure was used for the detection of cathepsin K and S immunoreactive polypeptides in the tissue extracts except that the primary antibodies were rabbit anticathepsin K (dilution, 1:10) and anticathepsin S (dilution, 1:50), respectively. The secondary antibody used for both was goat antirabbit IgG alkaline phosphatase (dilution, 1:5000) (Sigma).

Immunohistochemistry

Frozen specimens of spleen were sectioned at 4 μm, placed on 0.1% poly-L-lysine-coated slides, fixed in 100% acetone at −20°C for 15 minutes, and then fixed in acetone at room temperature for 15 minutes. Nonspecific binding was blocked using normal goat serum in PBS (dilution, 1:10) containing 0.1% wt/vol BSA. The cathepsin B protein was detected by using cathepsin B immune-specific antibody (dilution, 1:500) prepared in the same buffer and by incubating at room temperature for 60 minutes. Negative controls using either no primary antibody or preimmune serum (dilution, 1:500) were used to identify nonspecific staining. Immunoreac-
tive polypeptides were detected using swine antitissue fluorocine-
conjugated secondary antibody (dilution, 1:10) and incubating in the dark at room temperature for 1 hour. Cathepsin K protein was detected in a similar manner using cathepsin K antibody (dilution, 1:100) and goat antirabbit fluorocine-conjugated secondary antibody (Sigma) (dilution, 1:50). The slides were mounted using Cytofluor (ChemLab, Canterbury, England) in PBS and glycerol, and they were examined using an ultraviolet (UV) fluorescence microscope (Nikon, Japan).

Measurement of cathepsin activities

Activities of cathepsins B, K, and S were measured by the method of Barrett et al. with modifications. The fluorogenic substrates used were CBZ-B-Arg-AMC (pH 5.5) to measure cathepsin B and CBZ-F-Arg-AMC (neutral pH) to measure cathepsins K and S. We diluted 50 μL plasma, serum, or tissue extracts to 250 μL using 0.1% Brij-35 and preincubated the extracts with 250 μL incubation buffer containing 352 mM/L KH2PO4, 48 mM/L NaH2PO4, and 4 mM/L sodium EDTA (pH 6.0), containing 100 μM/L cysteine as an activator, at 37°C for 5 minutes. The assays were started by the introduction of 250 μL substrate (concentration, 0.02 mM/L; final concentration, 6.66 mM/L). After incubation at 37°C for 15 minutes, the reaction was stopped by the addition of 1 mL 100 mM/L sodium chloroacetate in a buffer containing 30 mM/L sodium acetate and 70 mM/L acetic acid (pH 4.3). Fluorescence emission at 460 nm was determined using a Perkin Elmer luminescence spectrophotometer L530 (Perkin Elmer, Courbevoie, France) after excitation at 380 nm. Cathepsin K activity was measured similarly, except we used a buffer
with 100 mmol/L sodium acetate (pH 5.5) containing 20 mmol/L cysteine and 5 mmol/L EDTA, and the substrate was CBZ-F-R-AMC. The same substrate was used to measure cathepsin S activity, except that the acetate buffer was adjusted to pH 7 with TRIS base. A fluorescence calibration curve was obtained with solutions of known concentrations of the released product, AMC. One unit of enzyme activity represents the amount of enzyme sufficient to hydrolyze 1 nmol substrate per minute at 37°C. The activity was expressed according to the protein concentration determined by the method of Bradford using a Coomassie reagent (Bio-Rad) and crystalline BSA as a standard and as described by the supplier.

**Statistical analysis**

All numerical data are expressed as the arithmetic means with standard deviations, unless otherwise stated. Statistical significance of the mean differences was examined by the Student t test and Lord’s range test for small numbers, where applicable.20

**Results**

**Differential gene expression in Gaucher spleen**

The Rsal–digested cDNA species differentially expressed in Gaucher spleen and obtained by suppression subtractive hybridization followed by amplification in the PCR was resolved by electrophoresis in agarose gels and visualized by ethidium bromide staining under UV light (Figure 1A). As shown in the subtracted population (lane 2), at least 30 visible cDNA fragments were present in this population obtained from the diseased spleen. This contrasts strikingly with the cDNA population not subject to selection (lane 1). Also depicted are the results of model subtractive hybridization experiments conducted with human muscle cDNA populations to which HaeIII–digested φ-X 174 DNA fragments had been added. As shown in the figure, this confirms the ability of the procedure to identify dominant DNA species present in the tester population of amplified skeletal muscle cDNA fragments (lanes 4 and 5).

To determine whether the procedure would permit the identification of genes already known to be differentially expressed in Gaucher tissue, the cDNA fragments were transferred to nylon filters by Southern blotting and hybridized with cDNA oligonucleotide probes for human chitotriosidase11,21,22 and type 5 acid phosphatase (Acp 5),23 whose expression is known to be enhanced in Gaucher tissue. As depicted also in Figure 1B,C, strong hybridization signals were obtained in the subtracted population of cDNAs with both gene probes; in the unsubtracted population, there was no signal obtained after prolonged film exposure, thus confirming the ability of the subtractive procedure to enrich for the products of genes known to be differentially expressed in Gaucher tissue.

**Products of the cDNA subtraction procedure**

In all, 64 cDNA fragments obtained from the PCR-subtracted cDNA population were sequenced. The sequences of 5 known genes encoding lysosomal proteins were identified, with an additional 10 cDNAs derived from known genes; 2 cDNA sequences representing hitherto uncharacterized human genes were also obtained. The sequences of the genes encoding lysosomal proteins showed an absolute sequence identity with human α-fucosidase, lysosomal acid lipase, and the human cathepsins B, K, and S.24-30

The cognate polypeptides derived from the 10 other known sequences showed identity with mucin core glycoprotein (MCG)24,31 the NMB protein previously identified in malignant melanoma,32 and the heparin sulfate proteoglycan (HSPG).33 In addition, 2 cDNA sequences with identity to human immunoglobulin-κ protein JC-κ of B-cell progenitors,34 and an IgG-κ immunoglobulin gene fragment and a cDNA sequence with identity to human β₂-microglobulin were also identified. In the population of known genes, a further sequence of interest was obtained by encoding a recently identified protein in the human C-C chemokine class, PARC, that shows closest homology to the human macrophage inflammatory protein-1–α (MIP-1–α).35

Three cDNA fragments encoding presumptive housekeeping genes were also identified in the selected population; these encode mitochondrial cytochrome oxidase and a ribosomal protein. The 2 novel cDNA fragments identified in the cDNA population obtained from Gaucher spleen show complete identity with expressed sequence tags. One is known to map to human chromosome 13, and both were derived from macrophage cell lines.36

**Studies of mRNA abundance in Gaucher tissue**

To determine whether the cDNA sequences identified within the subtracted population were up-regulated in Gaucher spleen, we carried out Northern blotting studies to examine the abundance and size of the expressed transcripts (Figures 2 and 3). These studies were conducted with RNA obtained from Gaucher spleen samples and control spleen samples other than those used in the original cDNA selection procedure. To control for mRNA transfer and quality on the filter tracks, Northern hybridization was carried out with radio-labeled mouse β-actin cDNA or human glyceraldehyde phosphate dehydrogenase cDNA to provide control signals that compensate for loading. Greatly increased signal intensities of transcripts hybridizing to the chitotriosidase and human tartrate-resistant Acp 5 of 1.7-kilobase (kb) and 1.5-kb probes, respectively, were found as expected (Figure 2).

In Figure 3, representative Northern blots demonstrate up-regulated signals for transcripts hybridizing to the human cathepsin B, K, and S cDNA probes compared with the signals obtained with loading control cDNAs. The apparent size of the transcripts for cathepsin B, at 5 kb and 4 kb, was unexpected because the transcript size in most tissues has been
reported to be 2.2 kb. However, multiple large transcripts of cathepsin B have been reported in human osteoclastoma and melanoma tissues. The hybridization signals for cathepsin K and human cathepsin S, at 1.8 kb and 1.3 kb, respectively, correspond to those previously reported as the dominant transcripts in human tissues. Northern blotting experiments also confirmed striking increases in the expression of the human chemokine PARC as well as the novel gene NMB and chromosome 13–related expressed sequence tag (EST) fragment transcripts of sizes corresponding to those previously reported for these expressed human genes, as described above. The relative abundance of these transcripts in Gaucher spleen RNA was compared with control spleen RNA. Steady-state enhanced expression of these genes was estimated to be increased at least 10-fold; in the case of cathepsins B and K, there was no signal detected on prolonged exposure of control spleen RNA samples to the hybridization probe, whereas intense signals were demonstrated in Gaucher spleen samples.

### Tissue distribution of differentially expressed genes

To determine the range of gene expression for genes not yet studied, Northern blots using RNA samples obtained from a range of human tissues were hybridized to radio-labeled cDNA probes carried out for the PARC human chemokine, the NMB protein, and human EST mapping to chromosome 13 (not shown). Selective expression of the PARC chemokine was noted principally in tissues containing antigen-presenting cells including macrophages, ie, spleen, thymus, intestine, blood leukocytes, and lung. The distribution of expression of the other genes was widespread in human tissues (not shown).

### Detection of altered protein expression in Gaucher disease

**Immunoblotting for cathepsin antigens in spleen.** To determine the molecular forms of cathepsin B, K, and S polypeptides in splenic tissue and to estimate changes in protein abundance, samples of spleen homogenate from Gaucher disease patients and controls were subjected to SDS-PAGE under denaturing conditions, and the polypeptides were immobilized by transfer to PVDF membranes for specific antibody-binding studies (Figure 4). Electrophoretic protein analysis showed few reproducible differences in the principal polypeptides stained between the disease and control samples; this procedure confirmed the uniform integrity of the tissue samples used for analysis in this study. A single polypeptide, approximately 24 kd, was found to be reproducibly increased in all 4 Gaucher spleen samples compared with 3 controls (not shown). Peptide sequencing of this protein, which was isolated from the dried gel, confirmed the identity of this species as the human L-chain ferritin molecule. However, immunoblotting experiments conducted with polyclonal antisera specific to human cathepsins revealed marked differences in Gaucher tissue. Immune staining for cathepsins B, K, and S (Figure 4) was greatly enhanced in the samples of Gaucher spleen. Cathepsin B demonstrated an additional isofrom in the Gaucher spleen (MW, approximately 30 kd)
compared to the 3 immunoreactive species present in control spleens (MW, approximately 29 and 20-21 kd), respectively.

The abundance of immunoreactive cathepsin B in whole spleen homogenate was determined by enzyme-linked immunosorbent assay (ELISA), which confirmed the increased abundance of immunoreactive cathepsin B. In 2 control spleens the mean concentration was 0.17 ng/mg protein compared with 3.8 ng/mg protein in the 3 Gaucher spleen extracts (Table 1). Immunoblotting also showed increased immunoreactive mature cathepsin K (MW, approximately 29 kd) and more procathepsin K (MW, approximately 38 kd) than in control tissue samples. Immunoreactive cathepsin S was also enhanced in Gaucher spleen, but it occurred as a single polypeptide species (MW, approximately 24 kd); this species was barely detectable in control spleen extracts.

**Immunohistochemical studies.** Having demonstrated enhanced expression of cathepsin RNA transcripts and polypeptides in Gaucher spleen, the cellular localization of the enhanced expression of antigen was examined by immunofluorescence microscopy using human isozyme-specific cathepsin antisera. To orientate these studies, sections of Gaucher spleen were stained with antihuman CD68 monoclonal antibody that recognizes antigens expressed on human macrophages (Figure 5). Immunofluorescence microscopy with cathepsin B-specific antisem showed marked staining in the sinusoidal endothelium of Gaucher spleens as well as localized staining within some Gaucher cells, as identified by their characteristic morphology and strong staining with anti-CD68. In contrast, cathepsin K antiserum reacted strongly and specifically with Gaucher cells, which demonstrated punctate staining; cathepsin K antigen was also stained in perisinusoidal lymphocytes and dendritic cells.

**Acid hydrolase activities in Gaucher spleen extracts**

Because increased expression of known and unexpected acid hydrolases was identified at the level of messenger RNA (mRNA) and protein antigen, studies were undertaken to determine their activities as well as the activities of known marker enzymes elevated in Gaucher disease (tartrate-resistant Acp 5) and chitotriosidase (Table 1). As expected, the mean activity of tartrate-resistant Acp 5 was increased approximately 10-fold in Gaucher spleen samples, and the mean activity of chitotriosidase was enhanced more than 30,000-fold in the Gaucher spleen extracts. The specific activities of cathepsins B, K, and S were determined by fluorimetric assays in Gaucher and control spleen extracts (Table 1). It is evident from the table that the specific activities of cathepsins B, K, and S were increased 7-fold to 23-fold in extracts of Gaucher spleen.

**Table 1. Acid hydrolases in human spleen**

<table>
<thead>
<tr>
<th></th>
<th>Control spleens (n = 3)</th>
<th>Gaucher’s spleens (n = 4)</th>
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<tbody>
<tr>
<td>Acp 5</td>
<td>2.6 ± 0.2</td>
<td>24 ± 13</td>
</tr>
<tr>
<td>Chitotriosidase</td>
<td>1.2 ± 0.7 × 10^3</td>
<td>3.8 ± 2.0 × 10^7</td>
</tr>
<tr>
<td>Cathepsin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>26 ± 4.1</td>
<td>183 ± 35</td>
</tr>
<tr>
<td>K</td>
<td>10 ± 2.0</td>
<td>97 ± 39</td>
</tr>
<tr>
<td>S</td>
<td>4.0 ± 2.1</td>
<td>91 ± 45</td>
</tr>
<tr>
<td>Immunoreactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cathepsin B</td>
<td>0.16, 0.18*</td>
<td>3.8 ± 0.9†</td>
</tr>
</tbody>
</table>

Measurements are given as nmol/min/mg protein (mean plus or minus SD), except for immunoreactive cathepsin B, which is measured as ng/mg protein. For Gaucher spleen, P < .01.

* Indicates n = 2.
† Indicates n = 3.

**Cathepsin activities in human blood**

Given the greatly increased enzymatic activities of cathepsins and known acid hydrolase marker enzymes in Gaucher spleen, it was important to determine whether or not these proteins were secreted into the plasma for enzymatic or immunochemical detection. Specific fluorimetric assays for cathepsins B, K, and S were conducted in plasma samples obtained from healthy subjects and in samples obtained from untreated Gaucher disease patients obtained at the time of diagnosis. As depicted in Table 2, the activities of all 3 cysteine proteinases were significantly enhanced in plasma obtained from patients with Gaucher disease.

**Cathepsin activities in other lysosomal storage diseases**

To determine the specificity of the elevated blood cathepsin activities, we assayed the following for cathepsin B, K, and S activities: samples of plasma from control subjects; patients with nonglycolipid lysosomal storage diseases (mucopolysaccharidoses, glycoproteinoses, and Batten’s disease); and glycosphingolipidoses other than Gaucher disease (Niemann-Pick diseases A and C, GM1 and GM2 gangliosidoses, Fabry’s disease, and Krabbe’s disease) as well as 11 additional untreated Gaucher disease patients at the time of diagnosis. Gaucher disease was
psins B, K, and S, respectively, in control sera (P the activities were 1.73
intact spleens were compared. In the 7 patients with intact spleens,
Gaucher disease patients treated by splenectomy and those with
remove the source of excess serum cathepsin activity, sera from
overexpression of cathepsins had been determined, would also
0.64
psins B, K, and S, respectively, in Gaucher sera compared with
6
Gaucher disease in all 6 patients examined.
resistant Acp 5 activities. As depicted in Figure 6, the activities of
as serum chitotriosidase, angiotensin-converting enzyme, or tartrate-
demonstrated clinical and objective improvements in disease
with either alglucerase or imiglucerase for 18-22 months and had
enzyme replacement therapy. All 6 patients had received treatment
samples from a 52-year-old man with Gaucher disease complicated
by severe osteoporosis. The samples were collected over a period of
16 months since he had started 30 IU/kg/mo enzyme therapy.
During the period of follow-up, an episode of radiologically
confirmed bone necrosis of the right femur developed. As shown in
Figure 7B, there was greatly increased activity of serum cathepsin
B before the onset of this bone crisis and a striking increase in
cathepsin K activity immediately after its onset. Similar behavior
was noted for cathepsin S (not shown in figure). These findings
strongly suggest that active cathepsins are released from Gaucher
tissue into the blood and that they reflect (and may predict) disease
activity at different stages of its evolution in the bone marrow.
The availability of ELISA for human immunoreactive cathepsin
B rendered it possible to investigate the presence of cathepsin B
protein in serum samples obtained from patients with Gaucher
disease. Serum from 11 control subjects contained 1.8 ± 1.7 ng
(mean plus or minus SD) of immunoreactive cathepsin B per mL,
and the serum from 10 untreated Gaucher disease patients con-
tained 11.5 ± 6.5 ng/mL, respectively (P < .01). The relative
increase (22-fold) in cathepsin B immunoreactivity in the spleen is
greater than the relative increase (7-fold) in measured enzymatic
activity compatible with the presence of more cathepsin B precu-
sors with lower specific activity. In the serum, cathepsin B
immunoreactivity was increased 6.4-fold but the absolute specific
activity of cathepsin B in the serum was reduced by about 500-fold,
which indicated that unlike the Gaucher spleen cathepsin B, most

Table 2. Cathepsin activities in human plasma

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cathepsin B</th>
<th>Cathepsin K</th>
<th>Cathepsin S</th>
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<tbody>
<tr>
<td>Gaucher's disease</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(n = 11)</td>
<td>2.12 ± 0.63*</td>
<td>5.11 ± 1.39*</td>
<td>5.63 ± 1.75*</td>
</tr>
<tr>
<td>Nonglycolipid storage</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>diseases (n = 8)</td>
<td>0.95 ± 0.12 (NS)</td>
<td>2.16 ± 0.47 (NS)</td>
<td>2.35 ± 0.90†</td>
</tr>
<tr>
<td>Glycosphingolipidases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 9)</td>
<td>1.97 ± 0.32 (NS)</td>
<td>2.93 ± 1.67 (NS)</td>
<td>4.25 ± 2.99†</td>
</tr>
<tr>
<td>Control subjects (n = 21)</td>
<td>1.17 ± 0.41</td>
<td>1.81 ± 0.46</td>
<td>1.32 ± 0.39</td>
</tr>
</tbody>
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Measurements are given as nmol/min/mL (mean plus or minus SD). NS indicates not significant (P > .05). * Indicates P < .05. † Indicates P < .01.

not receive enzyme therapy during several months of monitoring. The
sequential data demonstrated that although cathepsin K activities decreased progressively during treatment in patients who
received enzyme therapy, serum activities of cathepsin K remained
unchanged or increased slowly in the 3 mildly affected patients
who did not receive enzyme therapy (not shown). Enzyme therapy,
which has a beneficial effect specifically for Gaucher disease, also
reduces blood cathepsin activities. These results moreover suggest
that cathepsin release into the bloodstream may be correlated with
disease severity and treatment responses.

To determine further if serum cathepsin activities correlated
with disease severity, activities were examined in relation to the
clinical severity score index derived for 20 type I patients with
Gaucher disease.41 Serum B, K, and S activities were significantly
correlated with the severity score index (r = + 0.442, 0.599, and
0.472, respectively, P < .01). The relation between cathepsin K and
this clinical index is depicted in Figure 7A. Further information
about the relation between serum cathepsin activity and clinical
disease was provided by retrospective enzyme assays in serial
samples from a 52-year-old man with Gaucher disease complicated
by severe osteoporosis. The samples were collected over a period
of 16 months since he had started 30 IU/kg/mo enzyme therapy.

To investigate the disease-related specificity of our findings, serum
cathepsin activities were determined in stored sera obtained from 6
patients with Gaucher disease before and after introduction of
enzyme replacement therapy. All 6 patients had received treatment
with either alglucerase or imiglucerase for 18-22 months and had
demonstrated clinical and objective improvements in disease
activity as indicated by improvement in blood counts; visceral
volumes; symptoms; and other surrogate markers of disease such as
serum chitotriosidase, angiotensin-converting enzyme, or tartrate-
resistant Acp 5 activities. As depicted in Figure 6, the activities of
all cathepsins decreased in response to enzyme therapy for Gaucher
disease in all 6 patients examined.

In 3 patients, serial stored serum samples were available during the
institution of enzyme therapy and could be compared retrospectively
with serial samples obtained during the course of follow-up from 3 patients with mild symptoms of Gaucher disease who did

Figure 6. Serum cathepsin activities in patients with Gaucher disease before
and after enzyme replacement therapy. Serum samples from 6 patients with type I
Gaucher disease were assayed retrospectively before and 16-22 months after
institution of enzyme therapy with imiglucerase and when the principal manifestations
of the disease had regressed. The values for each patient were determined without
prior knowledge of treatment status and are depicted by individual symbols.
Cathepsins B, K, and S are shown in panels A, B, and C, respectively.
of the immunoreactive serum cathepsin B was catalytically inactive. This suggests that the cathepsin B is partially inactivated in serum by forming complexes with one or more circulating proteinase inhibitors such as α2-macroglobulin.

Discussion

Validation of the suppression subtractive hybridization method

The suppression subtractive hybridization procedure is based on the suppression of the PCR, which allows a normalization step to be carried out between the 2 populations of cDNA under study. This step is designed to overcome the wide differences in the abundance of individual transcripts between the 2 populations, thus facilitating the identification of a minority of differentially expressed genes distributed among numerous high-copy mRNA species. As shown in Figure 1, the suppression PCR method selectively suppresses amplification of abundant transcripts present in both populations and leads to a subtracted population containing a high frequency of up-regulated transcripts. 

Possible significance of cathepsins in Gaucher disease

Cathepsin B is an abundant lysosomal protein that displays diverse peptidase activities. Its physiologic role appears to be in the intralysosomal degradation of proteins; indeed cathepsin B is responsible for the activation of some lysosomal precursor enzymes as well as peptide prohormone processing. Cathepsin B is implicated in certain tissue destructive states including arthritis, bone resorption, and metastasis.

Possible significance of cathepsins in Gaucher disease

Evidence that the changes observed were not related to overt differences in tissue composition in the spleens examined and that they reflect Gaucher disease activity in other organs was provided by electrophoretic SDS-PAGE (not shown). This revealed closely similar polypeptide compositions and abundance between the 7 spleen samples used in this study. In addition, elevated cathepsin activities were found in sera obtained from patients with Gaucher disease, which was irrespective of splenectomy, including a pair of monozygotic twins homozygous for the N370S glucocerebrosidase allele; these were discordant for disease manifestations, and only one twin with overt disease had been treated by splenectomy. Elevated plasma and tissue cathepsin activities are directly related to the presence of Gaucher cells, as indicated by the following: (1) Gaucher cells in situ show strong immunostaining for cathepsin antigens. (2) Plasma activities of cathepsins B and K are not significantly elevated in patients with nonglycolipid lysosomal diseases or in patients with glycosphingolipidoses other than Gaucher disease. (Although cathepsin S activities may be elevated in these disorders, the relative increases are less striking.) (3) Specific therapy of Gaucher disease with macrophage-targeted human glucocerebrosidase correlated with a significant decrease in serum cathepsin activities. (4) Patients with symptomatic Gaucher disease showed a progressive decrease in their serum cathepsin activities toward normal levels. However, presymptomatic Gaucher disease patients not yet offered enzyme therapy had less elevated cathepsin activities which remained stable or increased gradually during prolonged monitoring, indicating a relationship between cathepsin activity and disease activity.

Possible significance of cathepsins in Gaucher disease

Cathepsin K has been recently identified as the principal
expressed protein of the osteoclast,\textsuperscript{38} and its pattern of expression is restricted to osteoclasts, the ovary, and colonic tissue.\textsuperscript{27} Cathepsin K is highly active in the cleavage of the bone matrix proteins collagen I and osteonectin and its role in bone resorption, modeling, and turnover is clearly demonstrated by the occurrence of an osteoprotropic syndrome in mice homozygous for a disrupted allele of cathepsin K.\textsuperscript{6} The role of cathepsin K in humans is vividly illustrated by the recent description of diverse mutations in the cathepsin K gene of patients with pycnodysostosis, a rare recessive trait characterized by osteosclerosis, short stature, skull deformities, and increased regions of demineralized bone, which indicate that the defective osteoclasts have impaired ability to degrade organic bone matrix.\textsuperscript{65} The development of specific inhibitors of cathepsin K activity is an active area of current pharmaceutical research.\textsuperscript{64} Given the intractable nature of the skeletal manifestations of Gaucher disease and the severity of the osteolytic lesions once established, enhanced cathepsin K expression associated with the condition immediately suggests the potential for selective cathepsin K inhibitors in this disorder.

Human cathepsin S is another cysteine proteinase whose expression was found to be greatly enhanced in the tissues, plasma, and serum of patients with Gaucher disease. Cathepsin S, like cathepsin K, shows a restricted pattern of tissue expression with the highest levels in the spleen, heart, and lung.\textsuperscript{65} In the latter tissue, detectable cathepsin S staining has only been identified in pulmonary alveolar macrophages, suggesting that the protein may have a specific role in the innate immune system including a role in antigen processing.\textsuperscript{47,66,67} Cathepsin S has diverse endopeptidase, di-peptidyl-peptidase, and amino-peptidase activities and a broad substrate activity range.\textsuperscript{66,70} Unlike other cathepsins, cathepsin S is stable and active at a neutral pH. Recent studies indicate that cathepsin S is highly expressed in lymphocytes, monocytes, and other major histocompatibility complex (MHC) class II–expressing cells, where it is readily induced by interferon-\gamma.\textsuperscript{71} The greatly increased activity of cathepsin S in Gaucher spleen and in the plasma of patients with this disorder is of interest because the protein may be involved in the abnormal immune regulation that characterizes chronic type I Gaucher disease.\textsuperscript{10,11}

Other macrophage–specific genes in Gaucher disease
The human C-C chemokine PARC has close homology to MIP-1\(\alpha\) and is abundant in pulmonary alveolar macrophages and follicular dendritic cells. This protein may contribute to the recruitment of lymphoid precursors and to B-cell activation, which characterize Gaucher infiltrates.\textsuperscript{8-11} Similarly, the cDNA fragment related to a human gene located on chromosome 13 and isolated from human macrophages indicates the extent to which macrophage activation is generalized in Gaucher tissue. The gene encoding the NMB protein may also merit further study because it is a tumor suppressor of unknown mechanism.

Pathways for gene activation in Gaucher disease
We and others have recently shown enhanced concentrations of proinflammatory cytokines including interleukin-6 (IL-6) in the serum of patients with Gaucher disease.\textsuperscript{8-11} We suggested that IL-6 hyperscrecretion may be a critical triggering factor for plasmacytoma and multiple myeloma in patients with Gaucher disease.\textsuperscript{10} IL-6 may also be a mediator of osteoclastic activation at the surface of the bone adjacent to infiltrating Gaucher cells. It is therefore significant that the human ACP5 gene encoding tartrate-resistant Acp-5 and the cathepsin K gene both harbour an IL-6 response element in their upstream 5′-untranslated region and cathepsin B transcription is stimulated by IL-6, which may represent a common pathogenetic pathway for phagocytic activation in Gaucher disease.\textsuperscript{72,73}

In summary, analysis of an enriched population of cDNA sequences that are enhanced in Gaucher disease has identified genes encoding lysosomal proteins which show selective expression in the plasma, serum, and tissues of Gaucher disease patients and, specifically, in their pathologic macrophages. The cluster of cDNAs representing cysteine proteases (cathepsins B, K, and S) have biologic functions that immediately suggest a pathogenic role. In particular cathepsin K, previously thought to be restricted in expression to the osteoclast,\textsuperscript{27,28,43,46,49} is highly active in Gaucher spleen and is expressed in the Gaucher cell.

The systematic identification of gene expression occurring as a result of glycolipid storage may ultimately improve methods for selecting patients at risk from complications of Gaucher disease and facilitate the development of protein markers with which to monitor its severity and long-term effects.

Acknowledgments
We thank Joan Grantham for preparing the manuscript, for secretarial assistance, and for supporting our Gaucher services. Dr John Grant kindly carried out the immunohistochemistry on fixed spleen sections. We are also indebted to the UK Gaucher Association for continued encouragement and support.

References


Pathologic gene expression in Gaucher disease: up-regulation of cysteine proteinases including osteoclastic cathepsin K

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